Cell, Vol. 123, 569–580, November 18, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2005.10.024

mTOR and S6K1 Mediate Assembly of the Translation Preinitiation Complex through Dynamic Protein Interchange and Ordered Phosphorylation Events

Marina K. Holz,[1,2] Bryan A. Ballif,[1] Steven P. Gygi,[1] and John Blenis[1,*]
[1]Department of Cell Biology
[2]Program in Biological and Biomedical Sciences
Harvard Medical School
Boston, Massachusetts 02115

Summary

In response to nutrients, energy sufficiency, hormones, and mitogenic agents, S6K1 phosphorylates several targets linked to translation. However, the molecular mechanisms whereby S6K1 is activated, encounters substrate, and contributes to translation initiation are poorly understood. We show that mTOR and S6K1 maneuver on and off the eukaryotic initiation factor 3 (eIF3) translation initiation complex in a signal-dependent, choreographed fashion. When inactive, S6K1 associates with the eIF3 complex, while the S6K1 activator mTOR/raptor does not. Cell stimulation promotes mTOR/raptor binding to the eIF3 complex and phosphorylation of S6K1 at its hydrophobic motif. Phosphorylation results in S6K1 dissociation, activation, and subsequent phosphorylation of its translational targets, including eIF4B, which is then recruited into the complex in a phosphorylation-dependent manner. Thus, the eIF3 preinitiation complex acts as a scaffold to coordinate a dynamic sequence of events in response to stimuli that promote efficient protein synthesis.

Introduction

Signal transduction pathways modulate cellular processes in eukaryotic cells in response to a variety of stimuli. The ability of a cell to respond to these stimuli depends in part on production of specific effector proteins. Traditionally, it was thought that the change in the proteome occurred as a result of transcriptional activity. However, we now know that regulating translation of specific messages is another way of altering protein production (Nilsson et al., 2004). The limiting step in the process of protein synthesis is translation initiation. Thus, it is important to understand how signal transduction pathways target and activate protein synthesis initiation.

The mammalian target of rapamycin, mTOR, has emerged as a critical nutritional and cellular energy checkpoint sensor and regulator of cell growth in mammalian cells (Gingras et al., 2001; Richardson et al., 2004; Schmelzle and Hall, 2000). This large protein serine/threonine kinase is a member of the PIKK family of protein kinases (Richardson et al., 2004) and, when complexed with the protein raptor, is very potently inhibited by the naturally occurring antifungal macrolide rapamycin (Kim et al., 2002). mTOR modulates the activity of two important translational regulators, the ribosomal S6 kinases (S6K1 and S6K2) and the eukaryotic initiation factor 4E (eIF4E), following changes in amino acid levels and energy sufficiency as well as stimulation by hormones and mitogens. These mTOR-regulated effectors control cell size and contribute to efficient G1 cell-cycle progression (Fingar and Blenis, 2004). Improper regulation of eIF4E and S6Ks contributes to carcinogenesis in cells with loss-of-function mutations in the tumor suppressors PTEN, TSC1/2, or LKB or upon gain-of-function mutations in many growth-factor receptors, phosphatidylinositol 3-kinase (PI3K), or Akt (protein kinase B) (Bjornsti and Houghton, 2004; Inoki et al., 2005; Tee and Blenis, 2005). In addition, inappropriate mTOR signaling can contribute to metabolic diseases such as diabetes and obesity (Hay and Sonenberg, 2004). Finally, more recent evidence has linked mTOR signaling to synaptic plasticity, memory, and general neuronal physiology (Kelleher et al., 2004).

mTOR regulates the function of eIF4E by regulating the phosphorylation of the eIF4E inhibitory proteins, 4E binding proteins (4E-BPs). Phosphorylation of 4E-BP1 promotes its dissociation from eIF4E bound to the mRNA 7-methylguanosine cap structure, allowing for recruitment of eIF4G and eIF4A. eIF3, 40S ribosomal subunits, and the ternary complex (eIF2/Met-tRNA/GTP) are also recruited to the cap, resulting in the assembly of the translation preinitiation complex (PIC) (for review, see Hay and Sonenberg, 2004). It is unclear how eIF4B, an essential regulatory subunit of the helicase eIF4A, is recruited to the PIC.

mTOR initiates S6K activation in response to cellular energy status, nutrient levels, and mitogens. Full and sustained S6K activation requires additional inputs by PI3K-dependent and -independent mechanisms and requires multiple growth-factor-induced phosphorylation events (Fingar and Blenis, 2004; Inoki et al., 2005; Martin and Blenis, 2002). Two essential phosphorylation sites include T229, located within the catalytic activation loop, and T389, located at the hydrophobic motif (reviewed in Martin and Blenis, 2002). S6K1 activation is initiated by mTOR/raptor-mediated phosphorylation of T389 (Kim et al., 2002), which requires the TOS motif located at the N terminus of S6K1 (Nojima et al., 2003; Schalm and Blenis, 2002). T389 phosphorylation creates a docking site for the phosphoinositide-dependent kinase 1 (PDK1), which then phosphorylates T229 (Alessi et al., 1998; Frodin et al., 2002). Following the initiating phosphorylation at T389 by mTOR, phosphorylation at this site is maintained by an agonist-regulated autophosphorylation mechanism (Romanelli et al., 2002). It is unclear how regulation of S6K1 by these multiple kinases is coordinated. We show that the eIF3 translation preinitiation complex serves an important function in organizing and coordinating this complex series of events.

An important question regarding S6K1 signaling is the molecular mechanics of how S6K1 coordinates the phosphorylation and function of proteins involved in regulating protein synthesis. S6K-mediated control of

*Correspondence: jblenis@hms.harvard.edu

translation was thought to occur through phosphorylation of the 40S ribosomal protein S6. The increase in S6 phosphorylation was proposed to govern the translation of a specific subset of mRNAs containing 5′-terminal oligopyrimidine (5′-TOP) tracts encoding ribosomal components and translation elongation factors (Jefferies et al., 1997); however, this model has been recently challenged (Pende et al., 2004; Tang et al., 2001). It now appears that S6K1, but not S6K2 and S6 phosphorylation, is linked to cellular growth control (Pende et al., 2004). Thus, the mechanism by which mTOR-regulated S6K1 mediates its effects on translation and cell growth remains a mystery.

One potentially important target of the S6 kinases in modulating protein synthesis is eIF4B, which is phosphorylated by S6Ks in vitro and in vivo (Gingras et al., 2001). The consequence of eIF4B phosphorylation on the process of translation initiation, however, remains unclear (Gingras et al., 2001). The S6Ks may also contribute to translation elongation, as they have been shown to phosphorylate eukaryotic elongation factor 2 (eEF2) kinase (Wang et al., 2001). Again, it is not clear how this contributes to translational regulation.

The eukaryotic translation initiation factor 3 (eIF3) is a complex consisting of at least 12 subunits (Mayeur et al., 2003). eIF3 interacts with the 40S ribosomal subunit as part of the 43S translation preinitiation complex and plays a role in eIF2/Met-tRNA/GTP ternary-complex association and mRNA binding (Gingras et al., 2001). The mRNA helicase complex, eIF4A/4B, also associates with the preinitiation complex and is important in unwinding complex 5′UTRs for efficient translation (Methot et al., 1996). Significantly, eIF3 subunits are frequently overexpressed in a variety of human cancers (Rajasekhar and Holland, 2004), which suggests a link between the aberrant regulation of translation initiation and neoplastic transformation.

Here, we present a connection between the mTOR/S6K1 pathway and translation initiation. We show that the eIF3 complex acts as a scaffolding platform that associates with mTOR and S6K1 in a growth-factor- and rapamycin-sensitive manner, with mTOR associating with the eIF3 translation preinitiation complex and S6K1 dissociating from the eIF3 complex upon hormone or mitogenic stimulation. This sequence of events promotes the coordinated phosphorylation and activation of S6K1 and the phosphorylation of the 40S ribosomal protein S6 and eIF4B, a process that was previously unclear. Phosphorylation of eIF4B then contributes to its association with the translation preinitiation complex, where it is predicted to partner with eIF4A to form a fully functional mRNA helicase (Rogers et al., 1999, 2001). The results presented here demonstrate a role for eIF4B phosphorylation in stimulating its recruitment to the translation preinitiation complex and uncover a regulated mechanism by which mTOR and S6K1 signaling is integrated into the process of translation initiation.

Results

Purification of eIF3-Complex Subunits as S6K1 Binding Proteins

In order to identify novel ways by which S6Ks might regulate protein translation, we sought to purify S6K1-associated proteins by employing the tandem-affinity purification (TAP) approach. We stably introduced pBABE vector expressing S6K1 with a dual HA and Protein A epitope tag into HEK293E cells and purified the associated proteins by a two-step procedure. S6K1-interacting proteins were eluted with $MgCl_2$ from the HA column in the second step of the purification as previously described (Ballif et al., 2004) and separated by SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie brilliant blue, revealing a number of proteins that interacted with S6K1 but not with Protein A alone (Figure 1A). Protein bands were excised and analyzed by mass spectrometry. Two of these proteins were identified as subunits p40 and p47 of the translation initiation factor eIF3 complex (eIF3h and eIF3f). The copurification of the eIF3-complex subunits was also confirmed by other immunoprecipitations in which we identified core subunits eIF3c and eIF3b, as well as eIF3e complexed with S6K1 in growing HEK293E cells (data not shown).

The Interaction of the eIF3 Complex and S6K1 Is Regulated by Hormones, Mitogens, Phorbol Esters, Amino Acids, and Rapamycin

After identifying members of the eIF3 complex in the S6K1 bound fraction by TAP, we set out to confirm this interaction and investigate its regulation. As shown in Figure 1B, HA-S6K1 coimmunoprecipitated with the core subunits b and c of the eIF3 complex under serum-starved conditions in HEK293E cells. Strikingly, this interaction was potently disrupted by insulin stimulation. Moreover, pretreatment with rapamycin restored the binding of eIF3 to S6K1.

We next verified that endogenous S6K1 exhibited the same regulated interaction with the eIF3 complex. Figure 1C shows that endogenous S6K1 coimmunoprecipitated with endogenous eIF3b under serum-starved and rapamycin-treated conditions but not after stimulation with insulin. The same pattern was observed when cells were stimulated with phorbol 12-myristate 13-acetate (PMA) or epidermal growth factor (EGF) and analyzed for the presence of S6K1 in eIF3b immunoprecipitates (Figure 1D). Again, the interaction observed under nonstimulated conditions was strongly disrupted by mitogen stimulation and restored by pretreatment with rapamycin. We also examined whether the S6K1/eIF3-complex interaction was observed in another cell type. As shown in Figure S1A in the Supplemental Data available with this article online, in serum-starved HeLa cells, S6K1 was associated with the eIF3 complex, and this interaction was disrupted by insulin addition and restored by rapamycin pretreatment. Thus, both endogenous and overexpressed S6K1 form a complex with eIF3 under conditions where S6K1 was not activated, and dissociation of S6K1 from the eIF3 complex by insulin, growth factors, or tumor-promoting phorbol esters was blocked by rapamycin. Finally, we determined whether amino acid availability, which also regulates mTOR signaling, modulated the S6K1/eIF3 interaction. As shown in Figure 1E, in nutrient-starved U2OS and HeLa cells, S6K1 was associated with the eIF3 complex, and this interaction was disrupted by amino acid addition and was restored by rapamycin pretreatment. Thus, nutrient availability also regulates the S6K1 and eIF3 interaction.

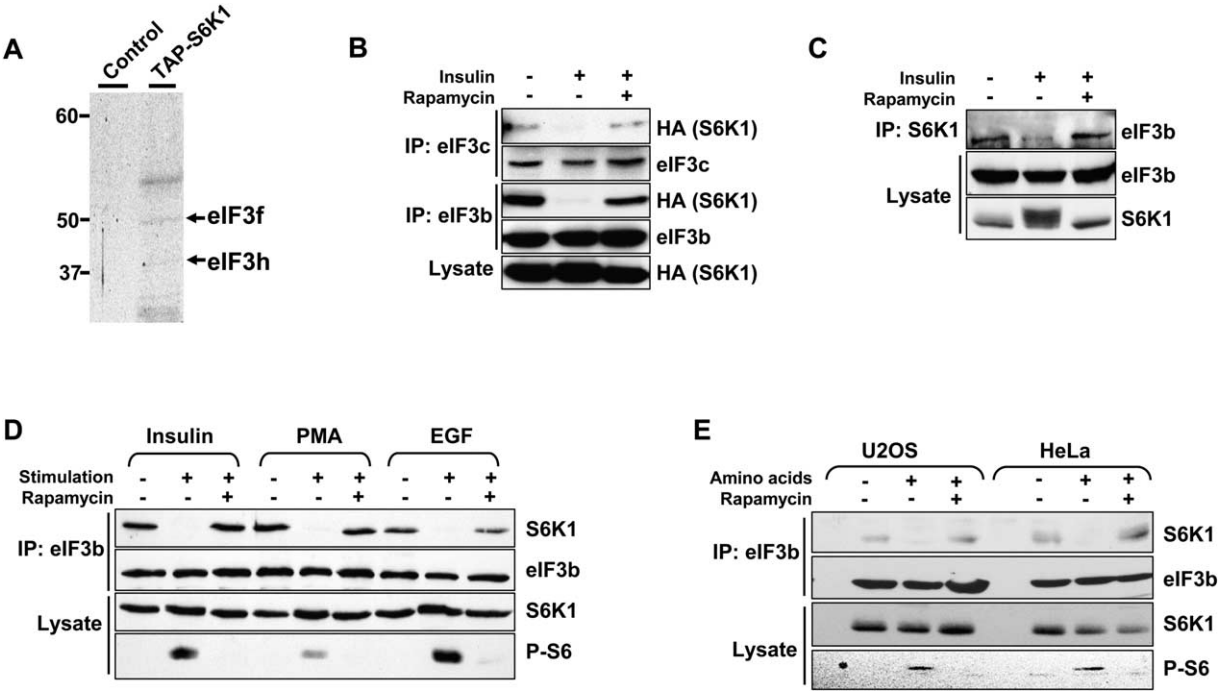

Figure 1. Purification of eIF3-Complex Subunits as S6K1 Binding Proteins and Identification of the eIF3 Preinitiation Complex as a Regulated Binding Partner of S6K1

(A) HA-tagged S6K1 was cloned in frame with the IgG binding domain of Protein A separated by a TEV cleavage site (TAP-S6K1). TAP elution was performed as described in Experimental Procedures, and eluants from control (Protein A alone) or S6K1 columns were resolved by SDS-PAGE. Arrows indicate the positions of S6K1-associated proteins eIF3f and eIF3h.

(B) Endogenous eIF3 core subunits interact with HA-S6K1. HEK293E cells were transfected with HA-S6K1, deprived of serum, stimulated, and lysed as described in Experimental Procedures. Endogenous eIF3c and eIF3b were immunoprecipitated, and their interaction with HA-S6K1 was determined by immunoblotting. Protein levels of eIF3b and eIF3c in immunoprecipitates and HA-S6K1 in the lysates are shown by immunoblotting with the respective antibodies.

(C) Endogenous S6K1 interacts with endogenous eIF3b. Endogenous S6K1 was immunoprecipitated and assayed for interaction with endogenous eIF3b. Protein levels of eIF3b in immunoprecipitates and S6K1 and eIF3b in the lysates are shown.

(D) Endogenous eIF3 and S6K1 interaction is regulated by various stimuli. Endogenous eIF3b was immunoprecipitated and assayed for interaction with endogenous S6K1 after stimulation with insulin, PMA, or EGF or pretreatment with rapamycin. Protein levels of S6K1 and eIF3b in immunoprecipitates and S6K1 and phospho-S6 (P-S6) in the lysates are shown.

(E) eIF3 interaction with S6K1 is regulated in U2OS and HeLa cells by addition of amino acids. Cells were starved of amino acids, stimulated, and lysed as described in the Experimental Procedures. eIF3b was immunoprecipitated, and the interaction with S6K1 is shown by immunoblot analysis. Levels of S6K1 and phospho-S6 (P-S6) in the lysates are shown. These data are representative of greater than three independent experiments.

Since our results suggested that the inactive, hypophosphorylated form of S6K1 associates with eIF3 and that activation correlates with its dissociation, we examined whether kinases that are involved in activation of S6K1 interact with the eIF3 complex. Specifically, we immunoprecipitated eIF3b from HEK293E cells and assayed for binding of endogenous Akt and PDK1. As depicted in Figure S1B, we found that Akt was not detected in eIF3b immunoprecipitates. Importantly, we also found that PDK1, which associates with the hydrophobic motif of S6K1 when it is phosphorylated (Figure S1C and Frodin et al., 2002), did not interact with eIF3 (Figure S1B). Therefore, the association between S6K1 and the eIF3 complex appears to be specific and does not extend to other closely related AGC kinases. Additionally, we were able to detect some HA-S6K2 binding to eIF3; however, under the conditions of our experiments, its association was not regulated by insulin or rapamycin (data not shown). Thus, only S6K1 demonstrates a regulated interaction with the eIF3 complex. To further characterize the association of S6K1 with eIF3, the eIF3:40S preinitiation complex, and polysomes, we separated these complexes by sucrose-gradient fractionation of cell extracts. Fractionation of extracts from cells incubated with insulin (Figure S2A) or insulin plus rapamycin (Figure S2B) revealed fractions in the low-density portion of the gradient that contained eIF3 but no 40S subunits, as determined by the absence of the 40S ribosomal protein S6; medium-density fractions containing eIF3 plus 40S subunits, representing preinitiation complexes; and high-density fractions containing little eIF3 but with 40S subunits as expected in polysomes. eIF3 and the 40S subunits failed to sediment to the high-density fractions if cells were treated with rapamycin (Figure S2B), as previously described (Miyamoto et al., 2005). We further analyzed fractions 4 and 5 (containing predominantly eIF3) and fractions 13 and 14 (containing preinitiation complexes) as depicted in Figure S2C and could only detect significant amounts of S6K1 to copurify with eIF3 in low-density fractions 4

and 5 after pretreatment with rapamycin. The lack of S6K1 in fractions 4 and 5 obtained from insulin-stimulated cell extracts demonstrated that this protein of approximately 70 kDa could not enter the gradient after its insulin-mediated release from the eIF3 complex. Therefore, S6K1 likely associates with eIF3-containing complexes in the absence of the 40S ribosome. Without a clearer understanding of the molecular basis for the interaction of S6K1 with eIF3 complexes in unstimulated or rapamycin-treated cells, however, we propose to define the eIF3 complex in our coimmunoprecipitation experiments as part of the eIF3 translation preinitiation complex, or eIF3-PIC.

The Phosphorylation of the Hydrophobic Motif at T389 Regulates the Interaction between S6K1 and the eIF3-PIC

The data presented above provide evidence that the activation state of S6K1 governs its binding to the eIF3-PIC. Thus, we tested an array of mutants of S6K1 for their ability to coimmunoprecipitate with the eIF3 complex. The positions of these mutations in S6K1 are diagrammed in Figure 2A. First, we examined the kinase-inactive (KD) mutant of S6K1 (containing a K100R mutation in the ATP binding site), the kinase-inactive TOS-motif mutant (F5A), and the kinase-inactive T229A mutant (containing a mutation at the phosphorylation site within the activation loop). As shown in Figure 2B, we found that, unlike the regulated interaction between wild-type S6K1 and eIF3, the kinase-inactive mutants of S6K1 bound constitutively to the eIF3 complex. The results with the F5A-S6K1 mutant also demonstrate that the binding of S6K1 to the eIF3 complex is not mediated by the TOS motif. These data are consistent with the observation that S6K1 phosphorylation, which correlates with its activation, promotes its dissociation from the eIF3-PIC.

Mutation of the TOS motif or the activation loop of S6K1 prevents phosphorylation of T389 (the hydrophobic motif), an important mTOR-regulated phosphorylation site necessary for its activation. We therefore hypothesized that the interaction between eIF3-PIC and S6K1 might be sensitive to mTOR-modulated S6K1 T389 phosphorylation. Thus, we investigated whether mutations at this site would affect the S6K1:eIF3-PIC interaction. The T389E mutation mimics phosphorylation of the hydrophobic motif and renders the kinase more active under basal conditions, still partially responsive to growth factors, and partially rapamycin resistant (see lower panel of Figure 2C and Schalm et al., 2005). As shown in Figure 2C, the T389E mutation greatly reduced eIF3 association with S6K1 under all conditions, similar to the behavior of activated S6K1. Importantly, the T389A mutation had the opposite effect compared to the T389E mutation: the T389A mutant constitutively bound to eIF3-PIC in a manner similar to the kinase-dead and F5A mutants. Therefore, the interaction between S6K1 and eIF3-PIC appears to be regulated by mTOR-dependent phosphorylation of S6K1 at T389.

We confirmed the contribution of S6K1 T389 phosphorylation to binding eIF3-PIC in the context of other mutations. We previously identified a stretch of three arginine residues in the C terminus of S6K1 that mediates an inhibitory effect on T389 phosphorylation, as mutation of these arginine residues to alanines (R3A) rescued the inhibition brought about by the F5A mutation and rendered the kinase rapamycin resistant (Schalm et al., 2005). As shown in Figure 2D, a combination of the F5A and R3A mutations allowed S6K1 to bind to eIF3b under basal conditions and dissociate from eIF3 after insulin stimulation. However, because the F5A-R3A mutant is rapamycin resistant, pretreatment with rapamycin did not restore the association between S6K1 and eIF3. Significantly, the combination of F5A-R3A and the hydrophobic-motif mutation T389E (F5A-389E-R3A) dramatically reduced S6K1 binding to eIF3, similar to T389E mutation alone. Finally, the K100R-T389E mutant, which is catalytically inactive but carries a phosphomimetic mutation at the hydrophobic motif, does not interact with the eIF3 complex (Figure 2E). Therefore, the activation of S6K1 is not required per se for dissociation of S6K1 from the eIF3-PIC scaffold. This interaction appears to be regulated by phosphorylation of T389.

The eIF3-PIC Interacts with mTOR/Raptor

Because our data pointed to the involvement of mTOR-mediated T389 phosphorylation in the ability of S6K1 to dissociate from eIF3-PIC, we tested the possibility that mTOR, in association with its binding partner raptor, would also be part of the eIF3-PIC. To test this hypothesis, HEK293E cells were transfected with AU-tagged mTOR and myc-raptor. Cells were lysed, and the complexes were immunoprecipitated using antibodies against the endogenous eIF3b and eIF3c. To our surprise, not only did mTOR and raptor coimmunoprecipitate with the eIF3 complex, but the interaction between mTOR/raptor and eIF3-PIC followed the pattern opposite of that of S6K1. Raptor and mTOR, which were weakly associated with eIF3 under serum-starved and rapamycin-treated conditions, bound strongly to eIF3c immunocomplexes following insulin stimulation (Figure 3A). Pretreatment with rapamycin reduced the insulin-mediated binding of mTOR/raptor to the eIF3 complex to basal levels. Similar results were obtained when immunoprecipitating endogenous raptor and mTOR (Figure 3B), although when monitoring endogenous proteins we noted lower levels of raptor binding in unstimulated or rapamycin-treated conditions. Therefore, upon insulin treatment, mTOR/raptor associates with eIF3-PIC, whereas S6K1 dissociates from the complex. To further characterize the interaction of mTOR with eIF3, eIF3:40S preinitiation complex, and polysomes, we separated these complexes by sucrose-gradient fractionation as shown and discussed for S6K1 (Figure S2). Importantly, unlike S6K1, we detected increased levels of mTOR copurifying with both low- and high-molecular-weight eIF3-containing complexes from cells incubated with insulin but not insulin plus rapamycin (Figure S2C; compare low-density fractions 4 and 5 to medium-density fractions 13 and 14). Taken together, these data suggest that the eIF3-PIC acts as a dynamic scaffold for mTOR and S6K1.

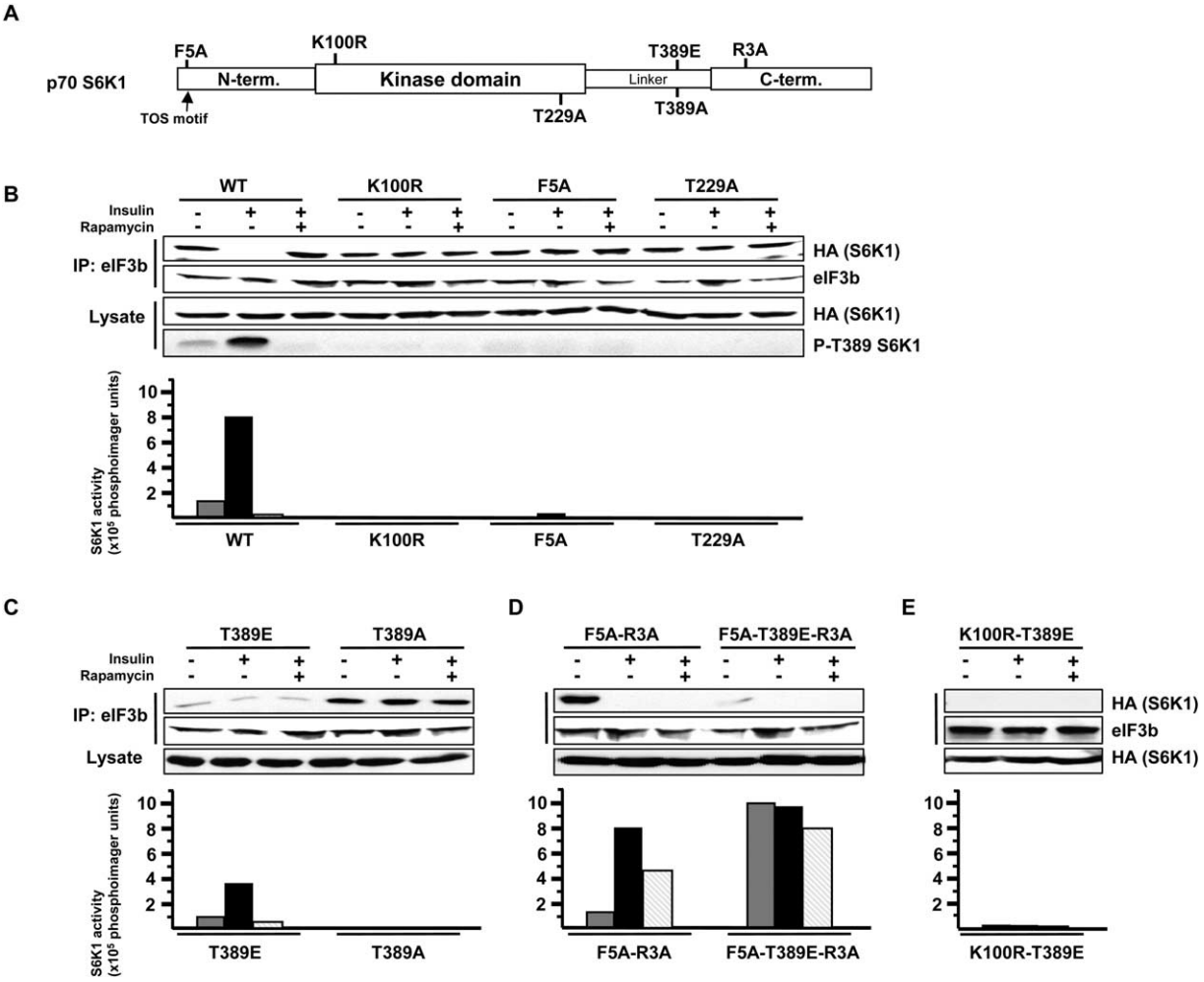

Figure 2. The Phosphorylation of the Hydrophobic Motif at T389 Regulates the Interaction between S6K1 and eIF3-PIC

(A) Schematic representation of the mutations in S6K1.

(B) Interaction of S6K1 mutants with eIF3b. HEK293E cells were transfected with wild-type (WT), F5A, K100R, and T229A alleles of HA-S6K1; deprived of serum; stimulated; and lysed as described in Experimental Procedures. eIF3b was immunoprecipitated and assayed for interaction with HA-S6K1 mutants by immunoblot analysis. Protein levels of eIF3b in the immunoprecipitates and HA-S6K1 and phospho-T389 in the lysates are shown by immunoblot analysis. The kinase activity of the S6K1 alleles toward GST-S6 was measured and quantified in the histogram.

(C) Same as in (B), with T389E and T389A alleles of S6K1.

(D) Same as in (B), with F5A-R3A and F5A-T389E-R3A.

(E) Same as in (B), with K100R-T389E. These data are representative of greater than three independent experiments.

The eIF3-PIC Interacts with S6K1 Targets

Since inactive S6K1 is docked at the eIF3 complex prior to agonist-induced translation initiation, we hypothesized that nutrients and mitogens serve to bring S6K1 near its translational targets. Hence, when activated, the tightly associated S6K1 is released from the eIF3 complex and is then able to phosphorylate these proximally associated proteins that regulate protein synthesis. To examine this possibility, we investigated whether the eIF3 complex associates with the 40S ribosome and eIF4B, which are targets of S6K1. As shown in Figure 4A, in addition to free eIF3, a portion of eIF3 was always associated with the 40S ribosome under our experimental conditions as monitored by immunoblotting eIF3 immunoprecipitates with antibody recognizing 40S ribosomal protein S6 (also see Figure S2), but increased S6 phosphorylation was only observed under conditions where S6K1 was phosphorylated and released from the eIF3-PIC. Interestingly, the association of eIF4B with the eIF3-PIC, as monitored by immunoblotting eIF3b immunoprecipitates with antibody recognizing eIF4B, was observed in insulin-stimulated but not serum-starved cells. Regulated association of eIF4B with the PIC had not been previously described. Furthermore, the insulin-stimulated interaction was potently disrupted by rapamycin treatment, suggesting that this interaction may also be regulated by post-translational events. Therefore, when S6K1 is activated, its release from the eIF3 complex correlates with S6 phosphorylation and the association of eIF4B.

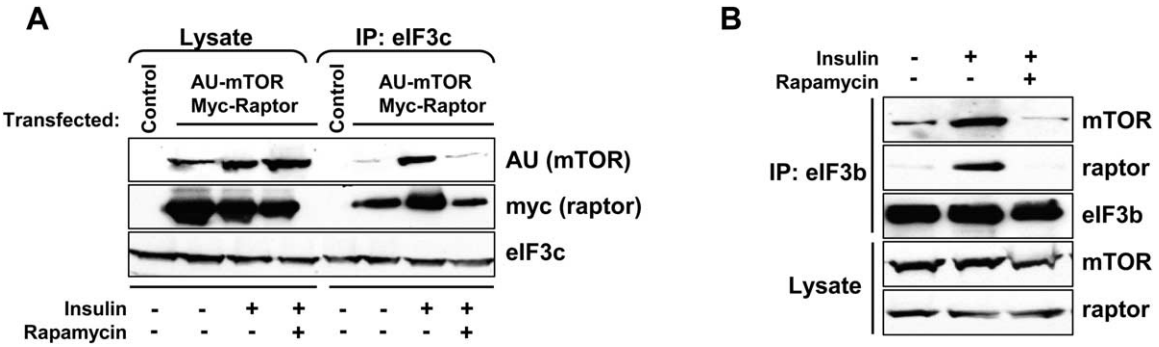

Figure 3. eIF3-PIC Interaction with mTOR/Raptor Is Positively Regulated by Insulin

(A) Transfected mTOR/raptor interacts with the eIF3-PIC. HEK293E cells were transfected with AU-mTOR and myc-raptor, starved, stimulated, and lysed as described in Experimental Procedures. eIF3c was immunoprecipitated and assayed for association with AU-mTOR/myc-raptor by immunoblotting. Protein levels in lysates and immunoprecipitates are shown by immunoblot analysis. Control is untransfected cells.
(B) Endogenous mTOR/raptor interacts with the eIF3-PIC. eIF3b was immunoprecipitated and assayed for association with mTOR/raptor by immunoblot. These data are representative of greater than three independent experiments.

Previous studies have been unable to determine a role for eIF4B phosphorylation. To test the hypothesis that phosphorylation of eIF4B by S6K1 leads to its recruitment to the eIF3-PIC, we used eIF4B phosphorylation mutants, nonphosphorylatable S422A, and phosphomimetic S422D. As depicted in Figure 4B, the S422A mutant was unable to associate with the eIF3-PIC under all conditions. Conversely, the S422D mutant constitutively bound to eIF3-PIC. Therefore, phosphorylation of eIF4B by activated S6K1 promotes its association with the translation preinitiation complex.

The T389A Mutant of S6K1 Prevents eIF4B Binding to the eIF3-PIC and the 7-Methylguanosine Cap Complex

We wanted to support the hypothesis that S6K1 activation and release from the eIF3-PIC was needed for subsequent phosphorylation of S6 and phosphorylation and association of eIF4B with the eIF3 complex. To do this, we overexpressed an inactive allele of S6K1 that is constitutively bound to eIF3-PIC to determine whether it would exhibit dominant-inhibitory activity and thus prevent binding or phosphorylation of S6K1 targets. As shown in Figure 4C, overexpression of T389A S6K1 inhibited the binding of eIF4B to eIF3-PIC after insulin stimulation. Phosphorylation of the 40S ribosomal S6 protein associated with eIF3 immunoprecipitates was also decreased when T389A S6K1 was expressed relative to phosphorylation of total cellular S6. We also determined whether T389A S6K1 overexpression blocked the recruitment of S6K1 targets to the 7-methylguanosine cap complex. As shown in Figure 4D, upon insulin treatment, T389A S6K1 prevented eIF4B binding to the 7-methylguanosine cap complex and decreased S6 phosphorylation. However, the binding of eIF4G, 4E-BP1, and eIF4E, which are not S6K1 targets, was unaffected. Importantly, the binding of eIF4G to the 7-methylguanosine cap complex correlated with the recruitment of mTOR/raptor and phosphorylation and release of 4E-BP1.

Temporal Association of Translational Components with eIF3-PIC and the 7-Methylguanosine Cap Complex

Our data suggested that, following growth-factor addition, mTOR/raptor associates with the eIF3 complex and phosphorylates and initiates activation of S6K1, which reduces its affinity for the complex and allows it to phosphorylate downstream targets such as 40S ribosomal protein S6 and the helicase subunit eIF4B. Phosphorylation of eIF4B then promotes its association with the translation preinitiation complex. To provide additional support for this model, we characterized the temporal regulation of endogenous protein binding to eIF3-PIC. First, we found that mTOR/raptor rapidly associated with eIF3-PIC within minutes of insulin stimulation. This correlated nicely with insulin-stimulated phosphorylation of S6K1 and its dissociation from the eIF3 complex (Figure 5A).

As shown in Figure 5A, maximal S6 phosphorylation lagged behind the phosphorylation and dissociation of S6K1 from the eIF3 complex. Treatment with rapamycin restored the binding of S6K1 to eIF3. Under these lysis conditions, the 40S subunit protein S6 appeared to coimmunoprecipitate with eIF3 throughout the time course. However, our sucrose-gradient fractionation analysis suggests that this represents a pool of free eIF3 and eIF3:40S subunits (Figure S2) as previously described (Miyamoto et al., 2005). Finally, we show that the binding of eIF4B with the eIF3-PIC correlated with its maximal phosphorylation, and also the maximal phosphorylation of S6—in other words, following phosphorylation, release, and the subsequent activation of S6K1 (Figure 5A).

We next investigated whether the association of proteins to eIF3-PIC occurred while it was docked at the cap complex. As shown in Figure 5B, we found that eIF3 was associated with the 7-methylguanosine cap complex with or without insulin, and in the presence of rapamycin, under our purification conditions. Previous studies have suggested that eIF3 is recruited to the 7-methylguanosine cap complex with eIF4G. We show, however, that overexpression of the TOS-motif mutant

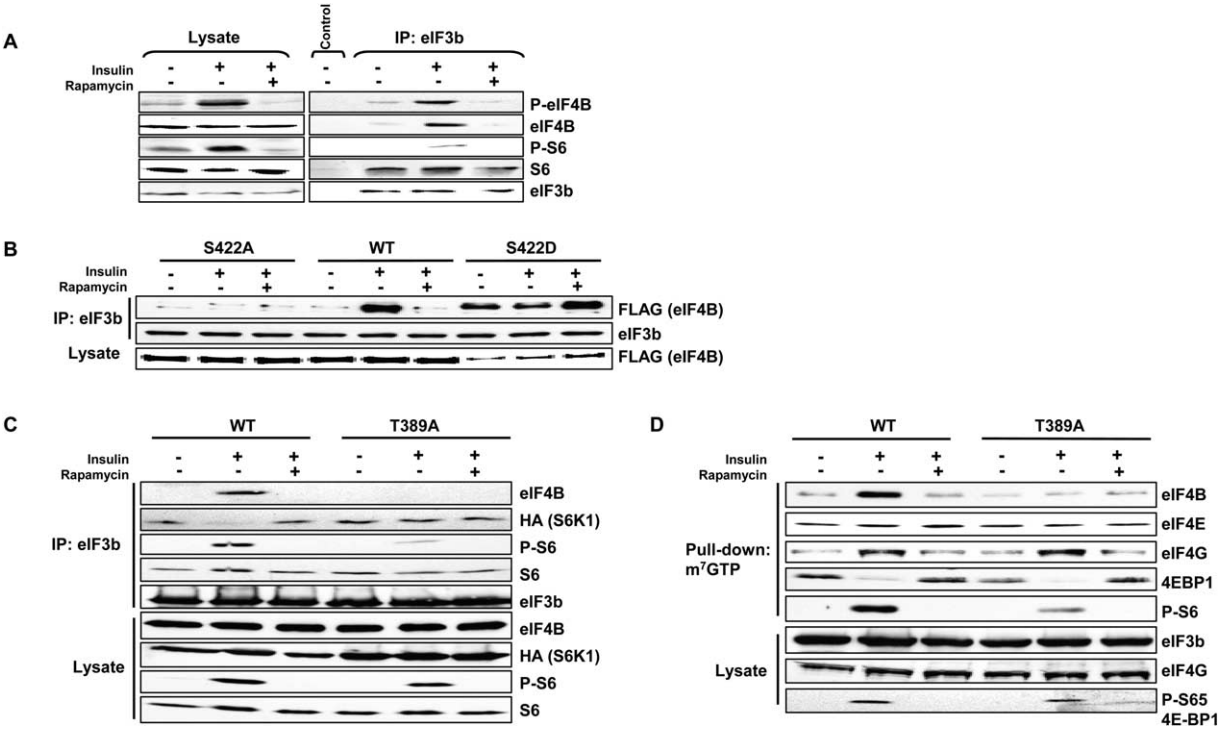

Figure 4. S6K1 Substrates S6 and eIF4B Interact with eIF3-PIC

(A) Endogenous eIF4B and S6 interact with eIF3-PIC. Endogenous eIF3b was immunoprecipitated from HEK293E cells and tested for interaction with S6 and eIF4B. The indicated protein and phosphoprotein levels were assayed by immunoblotting with their respective antibodies. Control is beads alone.

(B) eIF4B binding to eIF3-PIC is regulated by serine 422 phosphorylation. The indicated alleles of FLAG-eIF4B were transfected into HEK293E cells, and eIF3b was immunoprecipitated and blotted for interaction with eIF4B. Protein levels in lysates and immunoprecipitates are shown by immunoblot analysis.

(C) T389A S6K1 prevents eIF4B binding to eIF3-PIC. eIF3b was immunoprecipitated from cells transfected with wild-type (WT) or T389A mutant of S6K1 and assayed for interaction with eIF4B and S6. Protein and phosphoprotein levels in immunoprecipitates are shown by immunoblot analysis.

(D) Binding of eIF4B to the 7-methylguanosine cap complex is inhibited by T389A S6K1. Cells were treated as indicated, precipitated from cells transfected with wild-type (WT) or T389A mutant of S6K1, and assayed for interaction with the cap analog m^7GTP coupled to beads. Protein and phosphoprotein levels in precipitates and lysates are shown by immunoblot analysis. These data are representative of greater than three independent experiments.

of 4E-BP1 (F114A) (Schalm and Blenis, 2002; Schalm et al., 2003), which prevents its dissociation from eIF4E and therefore the association of eIF4G upon insulin stimulation, does not prevent the copurification of eIF3 with the cap beads (Figure S3A). Furthermore, both eIF4E and the eIF3 complex could be eluted from the cap structure with free m^7GTP (Figure S3C). We observed that eIF4G binds to the cap complex in time-dependent fashion upon insulin stimulation, whereas 4E-BP1 shows time-dependent dissociation from the cap, both of which were rapamycin sensitive. eIF4E, on the other hand, is constitutively present at the cap complex. These data suggest that eIF3 may interact with eIF4E. Given our data, we suggest a model where the multiprotein eIF3 complex possesses binding sites for eIF4E, eIF4G, mTOR/raptor, S6K1, and likely other proteins that regulate translation initiation. Future work will be needed to define the molecular basis for these interactions. The association of eIF3 with the preinitiation complex is likely stabilized following mTOR-dependent phosphorylation and release of 4E-BP1 and the subsequent recruitment of eIF4G. Importantly, in this assay, mTOR and eIF4B demonstrated time-dependent regulation of complex interaction upon insulin stimulation. These results are consistent with the model where recruitment of mTOR/raptor upon insulin stimulation not only serves to initiate S6K1 T389 phosphorylation and its dissociation, activation, and downstream signaling from the eIF3 complex but also results in bringing mTOR/raptor to the repressed eIF4E/4E-BP1 complex, where it phosphorylates 4E-BP1, thus promoting its dissociation. The scaffold protein eIF4G and associated proteins such as eIF4A then bind to the eIF4E/cap complex to stabilize the eIF3-PIC, recruit additional translation factors, and thus promote translation initiation.

Insulin, Rapamycin, and Members of the mTOR Signaling Pathway Regulate Cap-Dependent Translation

Finally, we set out to determine whether the mTOR pathway components contribute in vivo to cap-depen-

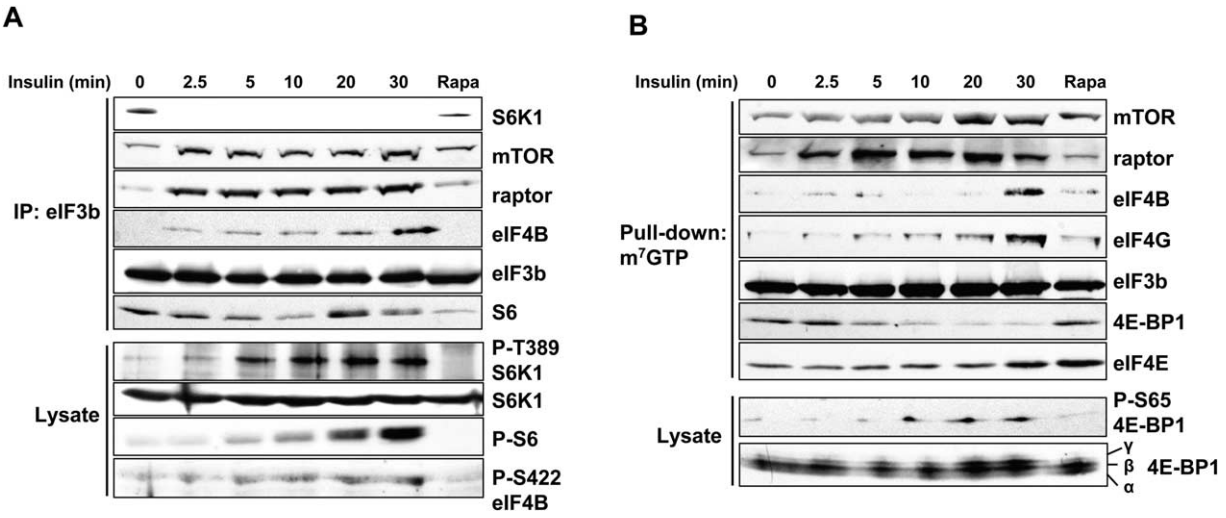

Figure 5. Temporal Association of Translational Components with the eIF3-PIC and the 7-Methylguanosine Cap Complex

(A) Time course of binding of S6K1, mTOR/raptor, eIF4B, and eIF4G to eIF3. Cells were treated with insulin as indicated or pretreated with rapamycin for 30 min (Rapa). Endogenous eIF3b was immunoprecipitated and assayed for interaction with the indicated endogenous proteins. Levels of proteins and phosphoproteins in the immunoprecipitates and lysates are shown.

(B) Time course of protein association with the cap structure. Cells were treated as in (A), and the indicated proteins were assayed for interaction with the cap analog m^7GTP. These data are representative of greater than three independent experiments.

dent translation. We used a dual-luciferase reporter system, depicted in Figure 6A, to address this question as described (Bordeleau et al., 2005; Kruger et al., 2001). Using this assay, we first measured the effect of insulin and rapamycin on cap-dependent translation. As shown in Figure 6B, insulin led to a 2-fold increase in cap-dependent over cap-independent translation rates in serum-deprived cells, and this effect was completely rapamycin sensitive. This suggested that the insulin-stimulated increase in cap-dependent translation was modulated by signaling through the mTOR pathway. To further define this observation, we measured the effect of mTOR on cap-dependent translation. As shown in Figure 6C, mTOR overexpression led to a dramatic increase in translation rates in growing cells. We next examined how overexpression of the downstream effectors of mTOR, eIF4E and S6K1, would affect cap-dependent translation. We observed that both eIF4E and S6K1 also enhanced cap-dependent translation relative to the control. To test our model that S6K1 association with the eIF3-PIC was important with regard to its role in translation initiation, we measured the effect of overexpressing the nonphosphorylatable T389A-S6K1 or F5A-S6K1 mutants, which bind constitutively to the complex. Overexpression of both mutants led to a decrease in translation, which was comparable to serum starvation or rapamycin treatment of growing cells. The use of both mutants also indicates that inhibition of translation was not due to competition of the overexpressed TOS motif within the T389A mutant with mTOR/raptor signaling to 4E-BP1, as the F5A mutant contains a TOS-motif mutation but still significantly interferes with translation. Overexpression of T389E mutant, which does not to bind the complex, did not have an effect on translation rates. Lastly, we tested the effect of eIF4B overexpression in this system. Overexpression of wild-type eIF4B enhanced cap-dependent translation. Additionally, overexpression of the mutant eIF4B S422D mimicking S6K1 phosphorylation had an even greater effect on cap-dependent translation. Overexpression of S422A-eIF4B mutant, which does not associate with the complex, had no effect on translation. Therefore, mTOR, S6K1, and eIF4B positively modulate cap-dependent translation.

Discussion

Until now, it was unclear how S6K1 participated in translation initiation. We have identified the eIF3-PIC as a dynamic scaffold for mTOR- and S6K1-mediated assembly of the translation initiation complex. The interaction between S6K1 and the eIF3-PIC is governed by the activating phosphorylation of S6K1 on the hydrophobic-motif residue T389. We have shown that phosphorylation of T389 is critical for the release of S6K1 from the eIF3 complex. However, it is not known whether T389 is a direct interface for interaction with this complex or whether the phosphorylation of T389 leads to a conformational change that disrupts S6K1 and eIF3-PIC binding. Importantly, other hydrophobic-motif-containing AGC-family kinases do not appear to interact with the eIF3-PIC in unstimulated or stimulated cells. Additionally, PDK1, which participates in hydrophobic-motif interactions, does not interact with this scaffold. This is of importance as PDK1 association with S6K1 has been reported to be enhanced following phosphorylation of the hydrophobic motif (Frodin et al., 2002). Once phosphorylated by mTOR/raptor at T389, S6K1 would be released, become accessible, and interact with PDK1, which would then promote phosphorylation of the S6K1 activation loop (T229) (see Figure 7). This would promote full activation of S6K1, and

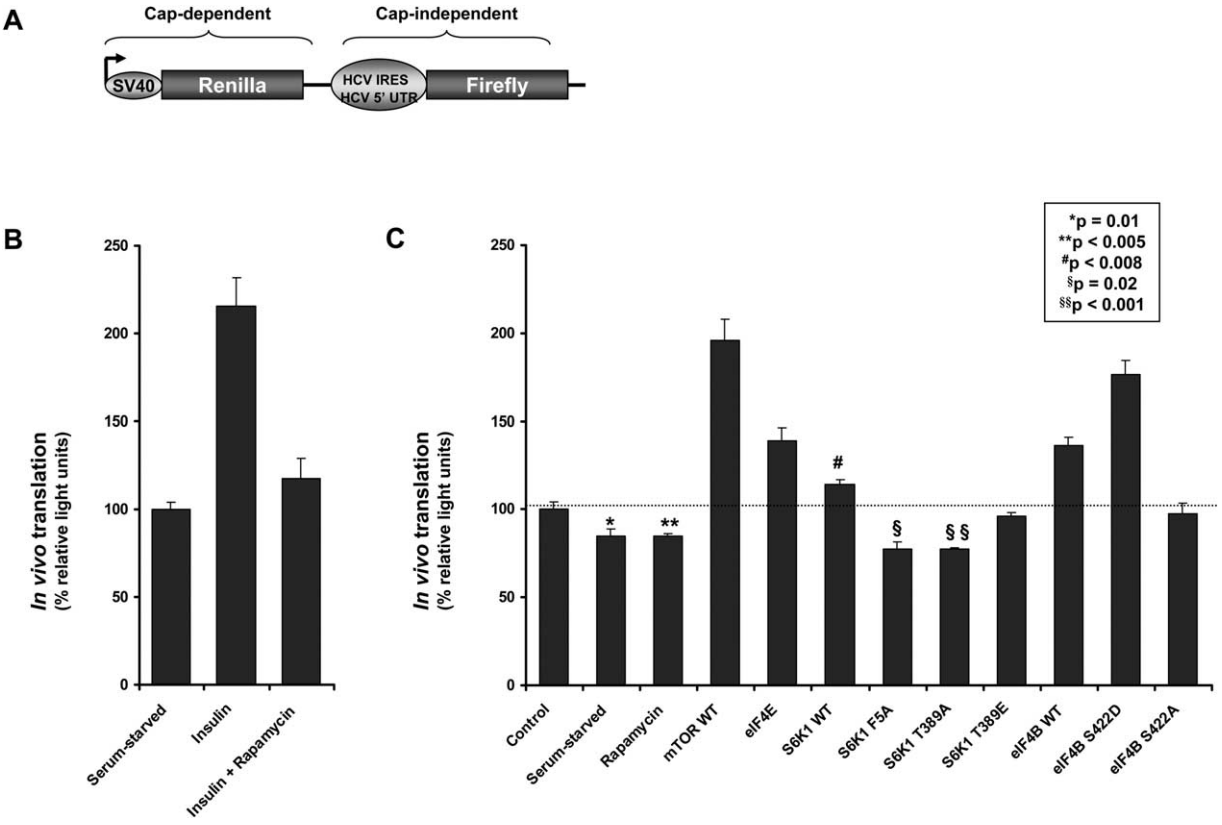

Figure 6. Insulin, Rapamycin, and Components of the mTOR Signaling Pathway Regulate Cap-Dependent Translation

(A) Structure of the bicistronic reporter plasmid allowing cap-dependent expression of renilla luciferase and expression of firefly luciferase dependent on HCV IRES.

(B) Cap-dependent translation is insulin stimulated and rapamycin sensitive. HEK293E cells were transfected in triplicate with the reporter plasmid and serum starved. Twenty-four hours posttransfection, cells were stimulated with insulin or pretreated with rapamycin and stimulated with insulin for an additional 24 hr. Luciferase activities were measured by a dual-luciferase assay, and the renilla/firefly luciferase light-unit ratio was calculated. The value of the serum-starved sample was set at 100%. Data are presented as the mean ± standard error.

(C) Overexpression of proteins in the mTOR pathway modulates cap-dependent translation. Cells were transfected with the bicistronic reporter plasmid and the indicated proteins. Twenty-four hours posttransfection, cells were grown for an additional 24 hr in 10% serum (control), serum-free media (serum-starved), or 10% serum in the presence of rapamycin. Cells transfected to express the indicated proteins were grown in 10% serum. Luciferase activities were measured as in (B). The value of the control was set at 100%. Data are presented as the mean ± standard error. Statistical significance was determined by Student's t test. *p = 0.01, §p = 0.02, §§p < 0.001, **p < 0.005, $^{\#}$p < 0.008; each data set was compared to the 100% control. The dashed line is drawn through the mean value of the control sample to facilitate comparison.

T389 phosphorylation could be maintained in the absence of a direct input from mTOR by a PDK1/PKCζ-regulated autophosphorylation mechanism as we have proposed (Romanelli et al., 2002).

The series of results obtained in this study allow us to postulate the following model for the regulated assembly and activation of an efficient translation initiation complex by mTOR/raptor and S6K1 (Figure 7). Under basal conditions, S6K1 is bound to the eIF3 complex, whereas mTOR association is greatly reduced. The interaction of mTOR and S6K1 with the eIF3 complex is dynamic and sensitive to serum starvation, insulin stimulation, and/or rapamycin treatment. The molecular details of this interaction have yet to be defined. The fact that rapamycin treatment reverses the effect of insulin or growth-factor treatment is important as it demonstrates that the observations made here are not simply related to starvation and stimulation conditions but are also relevant to conditions where the mTOR pathway is inappropriately activated and translation rates upregulated. For example, in cancers with activated PI3 kinase or loss of PTEN function, rapamycin should be very effective at reversing the biological consequences of inappropriately activated S6K1 signaling.

Thus, following an activating signal, such as insulin stimulation, mTOR/raptor is recruited to the eIF3-PIC, leading to phosphorylation of the bound and inactive S6K1 on T389. Phosphorylated S6K1 is then released from the eIF3 complex. Based on our sucrose-gradient profiles, 7-methylguanosine cap-bead pull-downs, and m^7GTP elution experiments, the eIF3 to which S6K1 binds appears not to be associated with 40S subunits and may be "free" and/or loosely associated with eIF4E bound to the mRNA 5′ cap. This observation has further significance in that it also places the recruited mTOR/raptor into position to phosphorylate 4E-BP1, the eIF4E bound suppressor of cap-dependent transla-

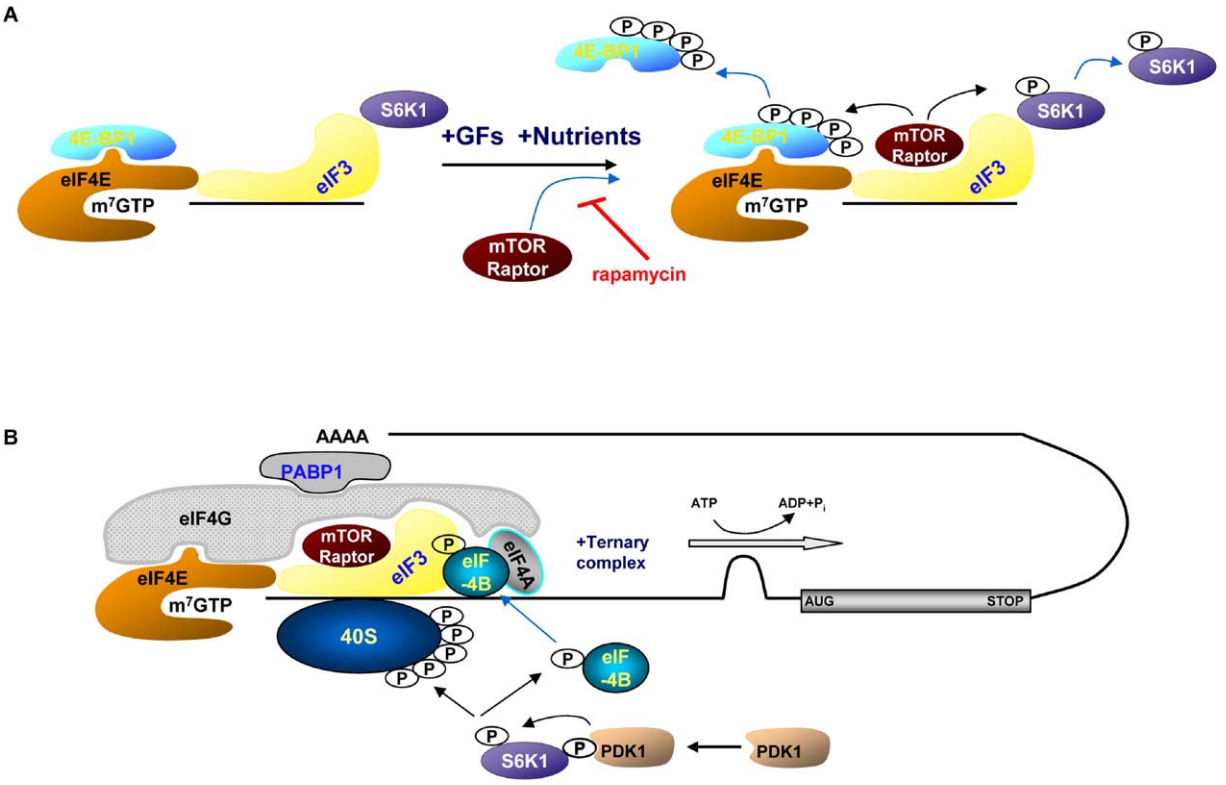

Figure 7. Model of Dynamic Interaction between the eIF3 Complex and mTOR/Raptor, S6K1, and eIF4B

Under basal conditions, S6K1 is associated with the eIF3 complex. Upon mitogen stimulation, an activation complex is formed in which mTOR/raptor is recruited to the eIF3 complex and phosphorylatea S6K1 and 4E-BP1. Phosphorylation of S6K1 at T389 leads to its dissociation from the eIF3 complex (A). T389-phosphorylated S6K1 then binds to PDK1, which phosphorylates S6K1 at T229. The fully activated S6K1 then phosphorylates eIF4B and S6 (B). Phosphorylation of eIF4B at S422 promotes its association with the translation preinitiation complex. Concurrently, mTOR/raptor phosphorylates 4E-BP1 (A), which dissociates from the cap complex, allowing recruitment of the eIF4G scaffold to the cap bound eIF4E as part of the eIF4F translation initiation complex (B). This sequence of events leads to preinitiation-complex assembly and initiation of protein translation.

tion. Thus the eIF3 complex acts as a scaffold allowing mTOR/raptor to promote the coordinated regulation of 4E-BP1 and S6K1 phosphorylation. Since PDK1 is not part of this scaffold complex, PDK1 must interact with T389-phosphorylated S6K1 after its dissociation, allowing for T229 phosphorylation within its activation loop and catalytic activation (Figure 7B). Activated S6K1 then phosphorylates eIF4B and the 40S ribosomal subunit protein S6. The S6K1 substrate eIF4B, when phosphorylated, is then recruited to the eIF3:40S ribosomal subunit preinitiation complex. The recruitment of phosphorylated eIF4B is not dependent upon the recruitment of eIF4G and the eIF4A helicase as the nonphosphorylatable 4E-BP1 (F114A) blocks insulin-stimulated recruitment of eIF4G to the cap complex without affecting recruitment of eIF4B. eIF4B recruitment is, however, antagonized by nonphosphorylatable mutants of S6K1, whereas eIF4G binding to the cap complex is not. Previous studies have shown that the helicase subunit eIF4A has low activity in the absence of eIF4B (Lawson et al., 1988; Rogers et al., 1999). Therefore, eIF4B phosphorylation by S6K1 provides a mechanism for recruitment of eIF4B to eIF4A at the translation initiation complex. The molecular details of how eIF4B and eIF4A then function together remains to be defined, but their corecruitment increases mRNA binding and processivity of the activated helicase complex, thus potentially enhancing translation rates of mRNAs with long, structured 5′ untranslated regions. Thus, eIF3, mTOR, and S6K1 coordinate the assembly of a translation initiation complex with enhanced translational capacity under conditions of nutrient and energy sufficiency that activate mTOR, combined with additional S6K1-activating inputs regulated by hormones such as insulin, growth factors, tumor-promoting phorbol esters, or a variety of oncogenic proteins (Fingar and Blenis, 2004; Martin and Blenis, 2002). Under these conditions, this translation initiation complex will be particularly efficient in the translation of mRNAs encoding proteins involved in ribosome biogenesis due to S6 phosphorylation (Jefferies et al., 1997), capped mRNAs due to mTOR-dependent phosphorylation of 4E-BP1 and recruitment of eIF4G (Fingar and Blenis, 2004), and mRNAs with long, complex 5′UTRs due to the phosphorylation-dependent recruitment of eIF4G (Fingar and Blenis, 2004; Gingras et al., 2001) and the eIF4A/B helicase complex (Hay and Sonenberg, 2004).

The increase in ribosome production combined with

the enhanced translation efficiency of mRNAs encoding proteins involved in G1 progression is necessary for cell growth and cell-cycle progression (Fingar and Blenis, 2004; Richardson et al., 2004). Therefore, elucidation of signaling interactions that feed into regulation of protein synthesis is important for understanding how cells respond to growth and proliferation cues and, when improperly regulated, contribute to a variety of diseases.

Experimental Procedures

Plasmids and Stable Cell Lines

pRK7-HA-S6K1 was described previously (Cheatham et al., 1995; Schalm et al., 2005). pcDNA3-AU-mTOR was provided by Robert Abraham (Burnham Institute, San Diego, California) and has been described (Brunn et al., 1997). pRK5-myc-raptor was provided by David Sabatini (MIT, Cambridge, Massachusetts) and has been described (Kim et al., 2002). The pCDNA3-FLAG-eIF4B constructs were provided by John Hershey (University of California, Davis, California) and have been described (Raught et al., 2004). Reporter plasmid pRL-HCV-FL was provided by Martin Kruger (Medizinische Hochschule Hannover, Hannover, Germany) and has been described (Kruger et al., 2001). pACTAG-2/3HA-BP1 was provided by Nahum Sonenberg (McGill University, Montreal), and generation of the F114A mutant has been described (Schalm et al., 2003).

The TAP vector was generated by cloning nucleotides 262–1104 encoding the first 340 amino acids of the *Staphylococcus aureus* Protein A followed by the TEV cleavage sequence into the BamHI, EcoRI sites of the pBABE vector. Protein A-S6K1 fusion was generated by in-frame ligation of HA-S6K1 into the EcoRI, SalI sites of the TAP vector. Stable cell lines were generated by transfecting the TAP vector or TAP-S6K1 into HEK293E cells, followed by selection in puromycin (Sigma).

Cell Culture, Transfection, Lysis, Coimmunoprecipitations, and Immunoblotting

HEK293E cells were cultured, transfected, and lysed for immunoblotting and coimmunoprecipitation as described previously (Martin et al., 2001). For experiments involving mTOR/raptor, HEK293E cells were cultured, transfected, and lysed for immunoblotting and coimmunoprecipitation as described previously (Schalm et al., 2003). After 20 hr of starvation in serum-free DMEM, cells were pretreated for 30 min with rapamycin (20 ng/ml) or ethanol vehicle and then stimulated with insulin (100 mM), EGF (50 ng/ml), or PMA (100 ng/ml) for 30 min. For amino acid starvation and restimulation experiments, after 20 hr of starvation in serum-free DMEM, U2OS or HeLa cells were starved for 1 hr in Dulbecco's PBS, pretreated for 30 min with rapamycin (20 ng/ml) or ethanol vehicle, and stimulated for 1 hr with MEM amino acid solution (GIBCO). Cell extracts were immunoprecipitated with the indicated antibodies for 2 hr, followed by incubation with 1:1 mixture of Protein A-Sepharose CL4B beads (Pharmacia) and Protein G beads (Sigma) for 1 hr. For cap binding assays, lysates were incubated with 7-methyl-GTP Sepharose (Amersham) for 2 hr. Immunoprecipitates were washed with lysis buffer. Proteins were resolved by SDS-PAGE, transferred onto nitrocellulose (Schleicher and Schuell), and probed with the indicated antibodies.

Antibodies

Anti-myc monoclonal antibodies were purchased from Charles River Laboratories. Anti-HA monoclonal antibodies were kindly provided by Margaret Chou (University of Pennsylvania, Philadelphia). Anti-AU1 mononoclonal antibodies were from Covance. Anti-phospho-S6 antibodies were kindly provided by Morris Birnbaum (University of Pennsylvania and HHMI, Philadelphia). Anti-eIF3, anti eIF4G, anti-mTOR, and 4E-BP1 antibodies were purchased from Santa Cruz Biotechnology. Anti-p70S6K antibody was described previously (Romanelli et al., 1999). Anti-Akt, anti-S6K1 T389, anti-S6, anti-eIF4E, anti-raptor, anti-4E-BP1 P-S65, anti-eIF4B, and P-S422 antibodies were purchased from Cell Signaling Technology. Anti-PDK1 antibodies were from Upstate USA, Inc. For immunoblotting, anti-rabbit, anti-mouse, and anti-goat horseradish peroxidase (HRP) conjugated antibodies were purchased from Amersham, Chemicon, and Santa Cruz Biotechnology, respectively.

Immune Complex Kinase Assay

S6K1 kinase assays were done as previously described (Romanelli et al., 1999).

Tandem-Affinity Purification

Ten 15 cm plates of stably transfected, growing cells were lysed, and lysates were incubated with IgG bead columns (Sigma) for 2 hr at 4°C. IgG columns were washed twice with 10 ml lysis buffer and equilibrated with 50 mM Tris (pH 8.0). TEV cleavage reaction was performed as described by the manufacturer (Invitrogen Life Technologies) for 2 hr at 4°C. The eluant was incubated with HA-antibody coupled to Affigel matrix (Bio-Rad) for 1 hr at 4°C. The matrix was washed with 10 ml lysis buffer. The associated proteins were eluted with 1 M $MgCl_2$, 50 mM HEPES (pH 7.2) followed by TCA precipitation. Precipitates were washed with acetone and resolved using 4%–12% SDS-PAGE.

Bicistronic Luciferase Assay

For luciferase reporter experiments, HEK293E cells were transfected with pRL-HCV-FL reporter plasmid (Kruger et al., 2001) and the indicated DNA. Forty-eight hours posttransfection, cells were harvested, and the luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega) and Turner Designs TD-20/20 luminometer according to the manufacturers' instructions.

Mass Spectrometry

Gel bands were excised, diced, and subjected to in-gel digestion with sequencing grade trypsin (Promega, 12.5 ng/μl) in 50 mM ammonium bicarbonate overnight at 37°C. Peptides were extracted with 50% acetonitrile (ACN), 5% formic acid (FA) and then dried. Peptides were then resuspended in 2.5% ACN, 5% FA and loaded using an autosampler onto a microcapillary column (100 μm × 12 cm) packed with reverse-phase MagicC18 material (5 μm, 200 Å, Michrom Bioresources, Inc.). Elution was achieved with a 5%–35% ACN (0.1% FA) gradient over 60 min, after a 20 min isocratic loading at 2.5% ACN, 0.5% FA. Mass spectra were acquired on a LCQ-Deca XP (Thermo Electron) over the entire run using eight MS/MS scans following each survey scan. Raw data were searched for fully tryptic peptides against the NCBI nonredundant human database using Sequest software and a mass allowance of 2 Da.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and three figures and can be found with this article online at http://www.cell.com/cgi/content/full/123/4/569/DC1/.

Acknowledgments

We thank members of the Blenis laboratory, especially Andrew Choo, Gregory Hoffman, and Philippe Roux, for helpful discussions and critical reading of the manuscript. We are especially thankful for the helpful advice and generosity of Drs. John Hershey and Nahum Sonenberg. This work was supported by National Institutes of Health grants GM051405 (J.B), CA046595 (J.B.), and HG00041 (S.P.G.).

Received: May 20, 2005
Revised: September 5, 2005
Accepted: October 31, 2005
Published: November 17, 2005

References

Alessi, D.R., Kozlowski, M.T., Weng, Q.P., Morrice, N., and Avruch, J. (1998). 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro. Curr. Biol. *8*, 69–81.

Ballif, B.A., Arnaud, L., Arthur, W.T., Guris, D., Imamoto, A., and

Cooper, J.A. (2004). Activation of a Dab1/CrkL/C3G/Rap1 pathway in Reelin-stimulated neurons. Curr. Biol. *14*, 606–610.

Bjornsti, M.A., and Houghton, P.J. (2004). Lost in translation: dysregulation of cap-dependent translation and cancer. Cancer Cell *5*, 519–523.

Bordeleau, M.E., Matthews, J., Wojnar, J.M., Lindqvist, L., Novac, O., Jankowsky, E., Sonenberg, N., Northcote, P., Teesdale-Spittle, P., and Pelletier, J. (2005). Stimulation of mammalian translation initiation factor eIF4A activity by a small molecule inhibitor of eukaryotic translation. Proc. Natl. Acad. Sci. USA *102*, 10460–10465.

Brunn, G.J., Fadden, P., Haystead, T.A., and Lawrence, J.C., Jr. (1997). The mammalian target of rapamycin phosphorylates sites having a (Ser/Thr)-Pro motif and is activated by antibodies to a region near its COOH terminus. J. Biol. Chem. *272*, 32547–32550.

Cheatham, L., Monfar, M., Chou, M.M., and Blenis, J. (1995). Structural and functional analysis of pp70S6k. Proc. Natl. Acad. Sci. USA *92*, 11696–11700.

Fingar, D.C., and Blenis, J. (2004). Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene *23*, 3151–3171.

Frodin, M., Antal, T.L., Dummler, B.A., Jensen, C.J., Deak, M., Gammeltoft, S., and Biondi, R.M. (2002). A phosphoserine/threonine-binding pocket in AGC kinases and PDK1 mediates activation by hydrophobic motif phosphorylation. EMBO J. *21*, 5396–5407.

Gingras, A.C., Raught, B., and Sonenberg, N. (2001). Regulation of translation initiation by FRAP/mTOR. Genes Dev. *15*, 807–826.

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. Genes Dev. *18*, 1926–1945.

Inoki, K., Ouyang, H., Li, Y., and Guan, K.L. (2005). Signaling by target of rapamycin proteins in cell growth control. Microbiol. Mol. Biol. Rev. *69*, 79–100.

Jefferies, H.B., Fumagalli, S., Dennis, P.B., Reinhard, C., Pearson, R.B., and Thomas, G. (1997). Rapamycin suppresses 5′TOP mRNA translation through inhibition of p70s6k. EMBO J. *16*, 3693–3704.

Kelleher, R.J., 3rd, Govindarajan, A., and Tonegawa, S. (2004). Translational regulatory mechanisms in persistent forms of synaptic plasticity. Neuron *44*, 59–73.

Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell *110*, 163–175.

Kruger, M., Beger, C., Welch, P.J., Barber, J.R., Manns, M.P., and Wong-Staal, F. (2001). Involvement of proteasome alpha-subunit PSMA7 in hepatitis C virus internal ribosome entry site-mediated translation. Mol. Cell. Biol. *21*, 8357–8364.

Lawson, T.G., Cladaras, M.H., Ray, B.K., Lee, K.A., Abramson, R.D., Merrick, W.C., and Thach, R.E. (1988). Discriminatory interaction of purified eukaryotic initiation factors 4F plus 4A with the 5′ ends of reovirus messenger RNAs. J. Biol. Chem. *263*, 7266–7276.

Martin, K.A., and Blenis, J. (2002). Coordinate regulation of translation by the PI 3-kinase and mTOR pathways. Adv. Cancer Res. *86*, 1–39.

Martin, K.A., Schalm, S.S., Romanelli, A., Keon, K.L., and Blenis, J. (2001). Ribosomal S6 kinase 2 inhibition by a potent C-terminal repressor domain is relieved by mitogen-activated protein-extracellular signal-regulated kinase kinase-regulated phosphorylation. J. Biol. Chem. *276*, 7892–7898.

Mayeur, G.L., Fraser, C.S., Peiretti, F., Block, K.L., and Hershey, J.W. (2003). Characterization of eIF3k: a newly discovered subunit of mammalian translation initiation factor eIF3. Eur. J. Biochem. *270*, 4133–4139.

Methot, N., Pickett, G., Keene, J.D., and Sonenberg, N. (1996). In vitro RNA selection identifies RNA ligands that specifically bind to eukaryotic translation initiation factor 4B: the role of the RNA remotif. RNA *2*, 38–50.

Miyamoto, S., Patel, P., and Hershey, J.W. (2005). Changes in ribosomal binding activity of eIF3 correlate with increased translation rates during activation of T lymphocytes. J. Biol. Chem. *280*, 28251–28264.

Nilsson, J., Sengupta, J., Frank, J., and Nissen, P. (2004). Regulation of eukaryotic translation by the RACK1 protein: a platform for signalling molecules on the ribosome. EMBO Rep. *5*, 1137–1141.

Nojima, H., Tokunaga, C., Eguchi, S., Oshiro, N., Hidayat, S., Yoshino, K., Hara, K., Tanaka, N., Avruch, J., and Yonezawa, K. (2003). The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. J. Biol. Chem. *278*, 15461–15464.

Pende, M., Um, S.H., Mieulet, V., Sticker, M., Goss, V.L., Mestan, J., Mueller, M., Fumagalli, S., Kozma, S.C., and Thomas, G. (2004). S6K1(–/–)/S6K2(–/–) mice exhibit perinatal lethality and rapamycin-sensitive 5′-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. Mol. Cell. Biol. *24*, 3112–3124.

Rajasekhar, V.K., and Holland, E.C. (2004). Postgenomic global analysis of translational control induced by oncogenic signaling. Oncogene *23*, 3248–3264.

Raught, B., Peiretti, F., Gingras, A.C., Livingstone, M., Shahbazian, D., Mayeur, G.L., Polakiewicz, R.D., Sonenberg, N., and Hershey, J.W. (2004). Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. EMBO J. *23*, 1761–1769.

Richardson, C.J., Schalm, S.S., and Blenis, J. (2004). PI3-kinase and TOR: PIKTORing cell growth. Semin. Cell Dev. Biol. *15*, 147–159.

Rogers, G.W., Jr., Richter, N.J., and Merrick, W.C. (1999). Biochemical and kinetic characterization of the RNA helicase activity of eukaryotic initiation factor 4A. J. Biol. Chem. *274*, 12236–12244.

Rogers, G.W., Jr., Richter, N.J., Lima, W.F., and Merrick, W.C. (2001). Modulation of the helicase activity of eIF4A by eIF4B, eIF4H, and eIF4F. J. Biol. Chem. *276*, 30914–30922.

Romanelli, A., Martin, K.A., Toker, A., and Blenis, J. (1999). p70 S6 kinase is regulated by protein kinase Czeta and participates in a phosphoinositide 3-kinase-regulated signalling complex. Mol. Cell. Biol. *19*, 2921–2928.

Romanelli, A., Dreisbach, V.C., and Blenis, J. (2002). Characterization of phosphatidylinositol 3-kinase-dependent phosphorylation of the hydrophobic motif site Thr(389) in p70 S6 kinase 1. J. Biol. Chem. *277*, 40281–40289.

Schalm, S.S., and Blenis, J. (2002). Identification of a conserved motif required for mTOR signaling. Curr. Biol. *12*, 632–639.

Schalm, S.S., Fingar, D.C., Sabatini, D.M., and Blenis, J. (2003). TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. Curr. Biol. *13*, 797–806.

Schalm, S.S., Tee, A.R., and Blenis, J. (2005). Characterization of a conserved C-terminal motif (RSPRR) in ribosomal protein S6 kinase 1 required for its mammalian target of rapamycin-dependent regulation. J. Biol. Chem. *280*, 11101–11106.

Schmelzle, T., and Hall, M.N. (2000). TOR, a central controller of cell growth. Cell *103*, 253–262.

Tang, H., Hornstein, E., Stolovich, M., Levy, G., Livingstone, M., Templeton, D., Avruch, J., and Meyuhas, O. (2001). Amino acid-induced translation of TOP mRNAs is fully dependent on phosphatidylinositol 3-kinase-mediated signaling, is partially inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation. Mol. Cell. Biol. *21*, 8671–8683.

Tee, A.R., and Blenis, J. (2005). mTOR, translational control and human disease. Semin. Cell Dev. Biol. *16*, 29–37.

Wang, X., Li, W., Williams, M., Terada, N., Alessi, D.R., and Proud, C.G. (2001). Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. EMBO J. *20*, 4370–4379.